Chemical Defense

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Novel Acetylenic Oxylipins from the Moss *Dicranum scoparium* with Antifeeding Activity against Herbivorous Slugs**

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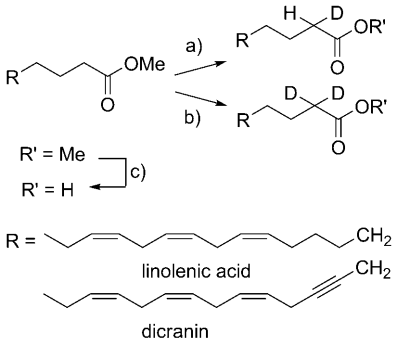
Dedicated to Professor Wilhelm Boland on the occasion of his 60th birthday

Mosses are seldom fed upon even in areas where intense herbivore pressure is observed on other plants. But even more than 100 years after the first investigations of Ernst Stahl on the chemical defense of plants and mosses against slugs and snails[1] we know very little about the nature and biosynthesis of metabolites responsible for moss chemical defense.[2,3] *Dicranum scoparium* is an example of a well-defended moss; it is globally distributed in temperate and arctic forests and often occurs in dense patches that show no sign of herbivory. In a recent survey of the volatile oxylipins (products of the oxidative transformation of polyunsaturated fatty acids) from mosses, we identified *D. scoparium* as a prolific producer of such metabolites.[4] In addition, this moss produces unusual acetylenic cyclopentenones from the dominant acetylenic fatty acid dicranin presumably also by means of oxylipin pathways.[5]

Motivated by the observation that oxylipins often serve as defense metabolites or regulators of defense reactions in higher plants,[6,7] we explored the chemistry and chemical ecology of oxylipins of *D. scoparium*. As observed in higher plants and diatoms, the production of volatile oxylipins is triggered by mechanical wounding in mosses as well.[6,8] Comparing the ultraperformance liquid chromatography/mass spectrometry (UPLC/MS) profiles of methanolic extracts obtained from *D. scoparium* before and after tissue disruption revealed that a complex mixture of metabolites is produced within seconds after wounding (Figure 1). Owing to the complexity of the chromatograms, we aimed to address the metabolic pathways guided by biosynthetic precursors labeled with stable isotopes. Therefore we prepared mono- and dideuterated fatty acids and administered them to the moss (Scheme 1).

The acetylenic fatty acid dicranin proved to be of special interest as a precursor of unusual novel oxylipins. After a 10 min administration of mono- or dideuterated dicranin suspended in water to frozen powdered moss, a complex mix of deuterated metabolites formed. Since not all oxylipins were chromatographically separated a manual evaluation of UPLC/MS data ran the risk of overlooking relevant signals. We therefore used an automated peak extraction routine that delivers mass/retention time pairs for every compound. These data from nontreated controls and mixtures obtained after treatment with mono- or dideuterated dicranin were then evaluated using a canonical analysis of principal coordinates[9] that enabled the rapid identification of dicranin-derived peaks (Figure 1; see the Supporting Information for experimental details). In contrast to the established manual evaluation of UPLC/MS runs this analysis allows an automated quick and comprehensive survey of metabolites derived from labeled precursors. This method covers the entire range of polarities and molecular weights recorded in LC/MS runs and picks up even minor or chromatographically not separated metabolites.



Scheme 1. Synthesis of mono- and dideuterated fatty aicds. a) LDA, quenching with MeOD; b) NaOMe/MeOD; c) KOH/H_2O.

The use of two precursors with different degrees of labeling in separate experiments supports the automated evaluation of the chromatogram since the second treatment serves as additional independent replicate (Figure 1c). The use of only one labeled precursor is also feasible, but more care has to be taken in the manual verification of the identified signals to avoid false positive hits. Mass/retention time pairs identified in the canonical analysis were used as guides in the purification of dicranin-derived metabolites using preparative HPLC. Structure elucidation of compounds **2**–**6** and **8** was based on one- and two-dimensional NMR as well as UV, IR, and MS data. The structure of **7** was tentatively assigned based on derivatization and GC/MS data.

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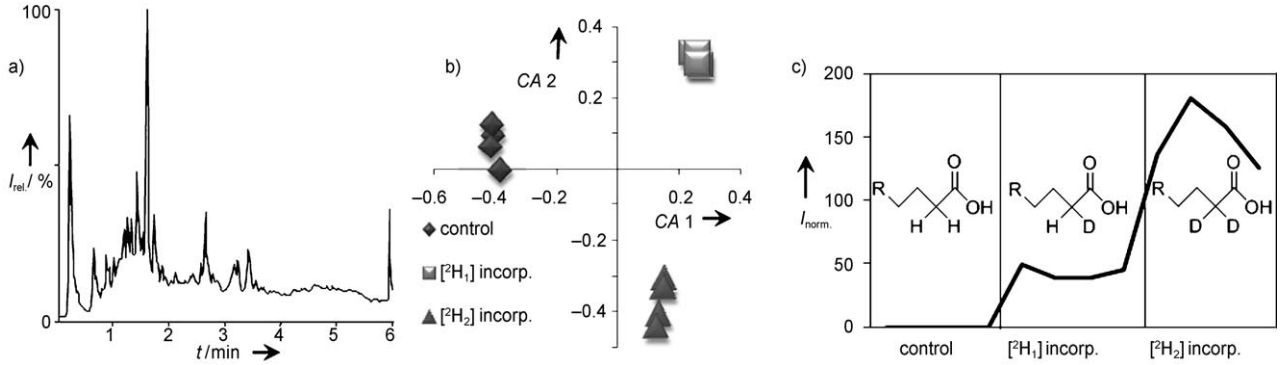


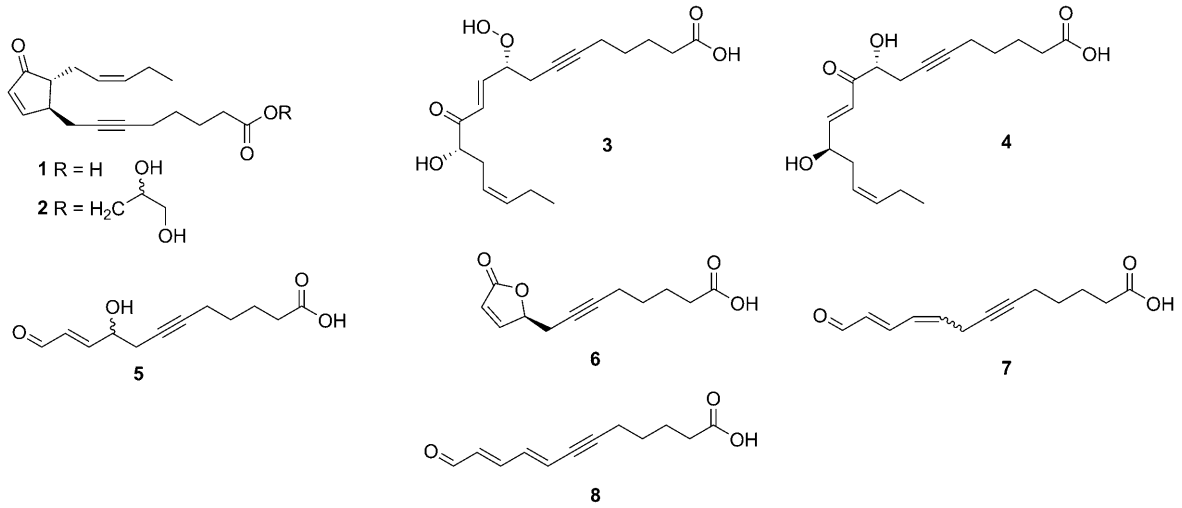
Figure 1. The search for dicranin-derived metabolites: a) UPLC/MS chromatograms (either control, addition of $[^2H_1]$- or $[^2H_2]$-labeled fatty acid) are evaluated by an automated peak extraction routine; $I_{rel.}$ = relative intensity normalized to the highest peak. b) Data of all replicates are processed by canonical analysis of principal coordinates to give a plot of the canonical axis (CA). Three separated groups are used to identify markers. c) Plot of one marker's normalized intensity $I_{norm.}$ for the different treatments; here the marker signal is 2 amu higher than the $[M-H]^-$ signal of the unlabeled metabolite.

Absolute configurations were determined using Mosher's method or by comparison of measured and calculated CD spectra (see the Supporting Information for structure elucidation and spectroscopic data). This protocol was used to identify seven novel dicranin-derived metabolites (Scheme 2).

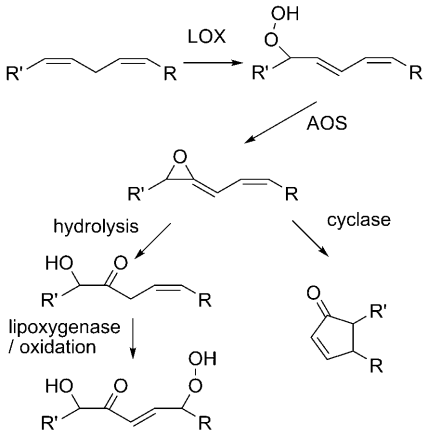
Among the known metabolites were the cyclopentenones dicranenone A (**1**) and B_1, which have antimicrobial properties.[5] The chiral dicranenones exhibited a high degree of labeling (up to 80%) after administration of the labeled fatty acids, indicating that all enzymes required for cyclopentenone production are active in wounded *D. scoparium*. We also detected the labeled monoacylglycerol **2**, which can result from the lipase-mediated esterification of de novo synthesized dicranenon A (**1**) with glycerol. In addition we identified the highly functionalized acyclic C_{18} oxylipins **3** and **4**. The (*E*)-5-hydroperoxy-1-hydroxypent-3-en-2-one structural motif as found in **3** is rare in nature and has been described so far only in a linolenic acid derived metabolite from flax seeds.[10] The (*E*)-1,5-dihydroxypent-3-en-2-one structural element observed in **4** has only been proposed for a linoleic acid metabolite from maize.[11] Both metabolites **3** and **4** are optically active, which indicates that at least the initial steps towards the formation of the α-ketols, but most likely also the further oxidation, are under enzymatic control (Scheme 3).

Administration of labeled fatty acids to the moss followed by canonical analysis was also used for the identification of linolenic acid derived metabolites as well (Scheme 4). Here, structure elucidation was based on GC/MS studies after derivatization and comparison with known standards (see the Supporting Information). While 12-oxophytodienoic acid (**9**) was found, the plant hormone jasmonic acid, which is derived from **9** in higher plants,[7] could not be detected in the moss. We also found significant amounts of the shorter chain length oxylipins **10**–**12**.

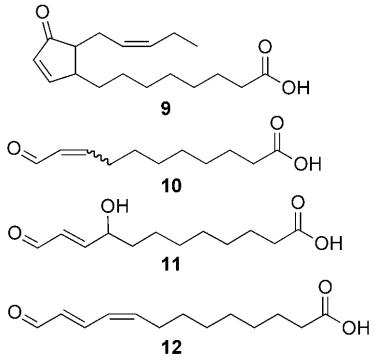
The biosynthesis of **1**–**12** could be initiated by lipoxygenase-mediated introduction of a hydroperoxide in the 9- or 13-position of the fatty acids. The resulting 9- or 13-hydro-



Scheme 2. New metabolites produced by the moss *D. scoparium* that arise from dicranin as the biosynthetic precursor.



Scheme 3. Biosynthetic pathways towards **1**–**4**. AOS = allene oxide synthase.



Scheme 4. Linolenic acid derived metabolites.

peroxides could be transformed by allene oxide synthases into intermediate allene oxides, which are cyclized or hydrolyzed to give **1**–**4** and **9** (Scheme 3). A hydroperoxide lyase or a lyase activity of lipoxygenases could catalyze the formation of **5**–**8** and **10**–**12**.[12] The observed formation of C_{12} and C_{13} metabolites from the C_{18} fatty acids would result in volatile C_6 and C_5 compounds as additional cleavage products. In accordance, 1-penten-3-one, (*E*)-2-pentenal, hexenol, and hexenal were found in the volatile bouquet of the moss after wounding.[4]

Using the purified metabolites as standards, the production of oxylipins was quantified by UPLC/MS. Only the phyllodes (green leafy structures) of the moss plants and not the stems are a source of oxylipins. Moss phyllodes contained up to 4.5 mg of the dominant metabolite **1** per gram of fresh weight.

Since such high amounts of oxylipins are produced and since several of the detected metabolites bear structural elements often associated with chemical defense,[6,8] we tested the feeding deterrent activity of crude lipid extracts and purified dicranenon A (**1**) against the herbivore *Arion lusitanicus* Mabille, a common slug in temperate regions.[13] Following a standard procedure for bioassays,[14] we sprayed defined amounts of the lipids or of purified metabolites dissolved in methanol on fresh lettuce leaves. Slug feeding on treated leaves was compared to feeding on control solvent-treated leaves in choice tests (see the Supporting Information for experimental details). The crude oxylipin-rich extract was highly active in natural concentrations, and significant activity was still observed when it was diluted 1000-fold (Figure 2). In

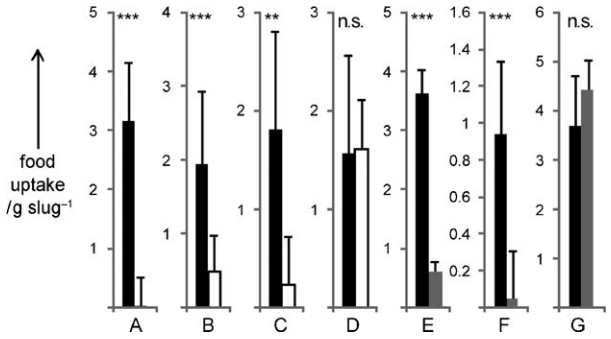


Figure 2. Feeding choice assays with lipid extracts from *D. scoparium* (A–D) and with purified dicranenone A (**1**; E–G). White and gray bars indicate feeding on lettuce treated with extracts and **1**, respectively. Solid bars indicate feeding on lettuce treated with the solvent methanol alone (controls). The crude oxylipin-rich extract is diluted 1:10 (A), 1:100 (B), 1:1000 (C), and 1:10000 (D) relative to the natural concentration. Purified dicranenone A (**1**) was employed in the natural concentration (E) and diluted 1:10 (F) and 1:100 (G). Error bars indicate standard error ($n = 15$). *** $p < 0.005$, ** $p = 0.010$, n.s. = not significant in t-test.

fact, the contribution of oxylipins to feeding deterrence might be even underestimated by this method since some of the reactive metabolites were unstable under assay conditions. Pure dicranenone A (**1**) was tested, and even this single component was active in natural and tenfold diluted concentrations. The activity of the pure compound as well as that of a highly diluted mixture of oxylipins illustrates a tremendous contribution of these compounds to the poor palatability of the moss.

This work demonstrates that the moss *D. scoparium* produces a diverse mixture of unusual oxylipins exhibiting pronounced chemical defense properties in choice assays with *A. lusitanicus*. Since the profiling of volatiles indicated that other mosses are also rich in oxylipins,[4] it can be speculated that in general oxylipins might significantly contribute to the effective chemical defense of mosses.

Experimental Section

A full description of the extraction procedures, chemoinformatic data treatment, spectroscopic data of the novel metabolites, and conditions of bioassays can be found in the Supporting Information.

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